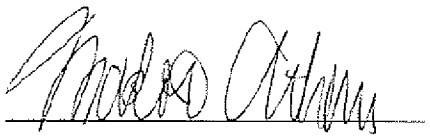
DECLARATION

I, Makoto AIHARA, Patent Attorney, of SIKs & Co., 8th Floor, Kyobashi-Nisshoku Bldg., 8-7, Kyobashi 1-chome, Chuo-ku, Tokyo 104-0031 JAPAN hereby declare that I am the translator of the certified official copy of the documents in respect of an application for a patent filed in Japan on January 30, 2002 under Patent Application No. 021159/2002 and that the following is a true and correct translation to the best of my knowledge and belief.

Dated: November 20, 2006



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[Name of Document] SPECIFICATION

[Title of Invention] GLYCOSYLATING ENZYME

[Claims]

[Claim 1] O-glycan α2,8-sialyltransferase, which is characterized in that it has the following substrate specificity and substrate selectivity.

Substrate specificity: the substrates of the enzyme are glycoconjugates having a Siaα2,3(6)Gal structure (wherein Sia represents sialic acid and Gal represents galactose) at the terminus thereof.

Substrate selectivity: the enzyme incorporates sialic acids into O-glycans more preferentially than into glycolipids or N-glycans.

[Claim 2] O-glycan α2,8-sialyltransferase having either one of the following amino acid sequences:

(1) an amino acid sequence shown in SEQ ID NO: 1; or

(2) an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and having O-glycan α2,8-sialyltransferase activity.

[Claim 3] O-glycan α2,8-sialyltransferase gene encoding the amino acid sequence of the O-glycan α2,8-sialyltransferase according to claim 2.

[Claim 4] The O-glycan α2,8-sialyltransferase gene according to claim 3 which has any one of the following nucleotide sequences:

(1) a nucleotide sequence corresponding to a portion between nucleotide 77 and nucleotide 1270 of a nucleotide sequence shown in SEQ ID NO: 2; or

(2) a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence corresponding to a portion between nucleotide 77 and nucleotide 1270 of the nucleotide sequence shown in SEQ ID NO: 2, and encoding a protein having O-glycan α2,8-sialyltransferase activity;

[Claim 5] A recombinant vector comprising the O-glycan α2,8-sialyltransferase gene according to claim 3 or 4.

[Claim 6] The recombinant vector according to claim 5 which is an expression vector.

[Claim 7] A transformant transformed with the recombinant vector according to claim 5 or 6.

[Claim 8] A method for producing the enzyme according to claim 1 or 2 wherein the transformant of claim 7 is cultured and the enzyme of claim 1 or 2 is collected from the culture.

[Claim 9] A protein which comprises an active domain of O-glycan α2,8-sialyltransferase having any one of the following amino acid sequences:

(1) an amino acid sequence corresponding to a portion between positions 26 and 398 of the amino acid sequence shown in SEQ ID NO: 1; or

(2) an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence corresponding to a portion between positions 26 and 398 of the amino acid sequence shown in SEQ ID NO: 1, and having O-glycan α2,8-sialyltransferase activity;

[Claim 10] An extracellular secretory protein, which comprises a polypeptide portion which is an active domain of the O-glycan α2,8-sialyltransferase of claim 1 or 2, and a signal peptide, and has O-glycan α2,8-sialyltransferase activity.

[Claim 11] A gene encoding the protein according to claim 9 or 10.

[Claim 12] A recombinant vector comprising the gene according to claim 11.

[Claim 13] The recombinant vector according to claim 12 which is an expression vector.

[Claim 14] A transformant transformed with the recombinant vector according to claim 12 or 13.

[Claim 15] A method for producing the protein according to claim 9 or 10 wherein the transformant of claim 14 is cultured and the protein of claim 9 or 10 is collected from the culture.

[Detailed Description of the Invention]

[0001]

[Industrially Applicable Field]

The present invention relates to a glycosylating enzyme and DNA encoding the enzyme. More specifically, the present invention relates to an enzyme (O-glycan α2,8-sialyltransferase, ST8Sia VI) that efficiently transfers sialic acid through an α2,8 linkage onto the sialic acid portion of a sugar chain having a Sia α2,3(6)Gal (Sia: sialic acid; Gal: galactose) structure at the terminus of O-glycans such as mucin, and DNA encoding the above enzyme. The O-glycan α2,8-sialyltransferase of the present invention is useful as a medicament having effects of suppression of cancer metastasis, prevention of virus infection, suppression of inflammatory response or activation of neural cells, as a reagent for increasing physiological action by adding sialic acid to a sugar chain, or as an enzyme inhibitor.

[0002]

[Prior Art]

Sialic acid is a substance responsible for important physiological actions such as cell-cell communication, cell-substrate interaction, and cell adhesion. The presence of sialic acid-containing sugar chains has been known, and some of such chains are expressed in stage-specific manner during development and differentiation, or in tissue-specific manner. Sialic acid exists at the terminal position of the sugar chain of a glycoprotein or glycolipid. Introduction of sialic acid into these sites is carried out emzymatically by transfer of sialic acid portion from CMP-Sia.

[0003]

Enzymes having a function in such enzymatic introduction of sialic acid (sialic acid tranfer) belong to a member of glycosyltransferases called sialyltransferases. So far, 18 types of sialyltransferases have been known with regard to mammals. These sialyltransferases are broadly divided into 4 families (Tsuji, S. (1996) *J. Biochem.* 120, 1-13). This is to say, these 4 families are: α2,3-sialyltransferase (ST3Gal-family) that transfers sialic acid onto galactose through an α2,3 linkage; α2,6-sialyltransferase (ST6Gal-family) that transfers sialic acid onto galactose through an α2,6 linkage; GalNAc α2,6-sialyltransferase (ST6GalNAc-family) that transfers sialic acid onto N-acetylgalactosamine through an α2,6 linkage; and α2,8-sialyltransferase (ST8Sia-family) that transfers sialic acid onto sialic acid through an α2,8 linkage.

[0004]

Of these, with regard to α2,8-sialyltransferase, cDNA cloning of 5 types of the enzymes (ST8Sia I-V) have been achieved so far, and their enzymatic properties have been elucidated (Yamamoto, A. *et al.* (1996) *J. Neurochem.* 66, 26-34; Kojima, N. *et al.* (1995) *FEBS Lett.* 360, 1-4; Yoshida, Y. *et al.* (1995) *J. Biol. Chem.* 270, 14628-14633; Yoshida, Y. *et al.* (1995) *J. Biochem.* 118, 658-664; Kono, M. *et al.* (1996) *J. Biol. Chem.* 271, 29366-29371). ST8Sia I is an enzyme for synthesizing a ganglioside GD3, and ST8Sia V is also an enzyme for synthesizing gangliosides GD1c, GT1a, GQ1b, GT3, and so on. ST8Sia II and IV are enzymes for synthesizing polysialic acid on the N-glycans of a neural cell adhesion molecule (NCAM). ST8Sia III is an enzyme for transferring sialic acid onto Siaα2,3Galβ1,4GlcNAc structures found in the N-glycans of glycoproteins and glycolipids. The preferred substrates for all of these enzymes are glycolipids or N-glycans. There have been only two reports in which these enzymes exhibit activity toward O-glycans. A case where ST8Sia II and IV synthesize oligosialic acid/polysialic acid on O-glycans found in an isoform of NCAM, and a case where ST8Sia III acts on the O-glycans of an adipocyte-specific glycoprotein AdipoQ (Suzuki, M. *et al.* (2000) *Glycobiology* 10, 1113; and Sato C, *et al.* (2001) *J. Biol.*

Chem. 276, 28849-28856). Thus, the previously reported α2,8-sialyltransferases do not generally utilize O-glycans as preferred substrates. The existence of α2,8-sialyltransferase which utilizes such an O-glycans as preferred substrates has been unknown.

[0005]

[Object to be Solved by the Invention]

As stated above, only 5 types of α2,8-sialyltransferases have been known so far. Main substrates for all of these enzymes are glycoproteins having N-glycans or glycolipids such as gangliosides. These enzymes show no activity toward glycoproteins having O-glycans, or show only a limited activity. It is the object of the present invention to solve this problem and to provide a novel O-glycan α2,8-sialyltransferase showing high activity toward O-glycans. It is also the object of the present invention to clone the cDNA encoding O-glycan α2,8-sialyltransferase, so as to provide a DNA sequence encoding the above O-glycan α2,8-sialyltransferase and an amino acid sequence of the above enzyme. Moreover, it is also the object of the present invention to allow a portion necessary for the activity of the above O-glycan α2,8-sialyltransferase to express as a protein in a large quantity.

[0006]

[Means for Solving the Object]

The present inventors have made intensive studies to achieve the above-described objects. The present inventors have screened mouse brain and heart cDNA libraries, and have also performed PCR using cDNA derived from mouse kidney as a template, so that they have succeeded in cloning the cDNA encoding O-glycan α2,8-sialyltransferase. Thus, the present invention has been completed.

That is to say, the present invention provides O-glycan α2,8-sialyltransferase, which is characterized in that it has the following substrate specificity and substrate selectivity.

Substrate specificity: the substrates of the enzyme are glycoconjugates having a Siaα2,3(6)Gal structure (wherein Sia represents sialic acid and Gal represents galactose) at the terminus thereof.

Substrate selectivity: the enzyme incorporates sialic acids into O-glycans more preferentially than into glycolipids or N-glycans.

[0007]

Preferably, the present invention provides O-glycan α2,8-sialyltransferase having either one of the following amino acid sequences:

(1) an amino acid sequence shown in SEQ ID NO: 1; or

(2) an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and having O-glycan α2,8-sialyltransferase activity.

[0008]

In another aspect of the present invention, the O-glycan α2,8-sialyltransferase gene encoding the above-described amino acid sequence of the O-glycan α2,8-sialyltransferase of the present invention is provided.

[0009]

Preferably, the present invention provides the O-glycan α2,8-sialyltransferase gene having any one of the following nucleotide sequences:

(1) a nucleotide sequence corresponding to a portion between nucleotide 77 and nucleotide 1270 of a nucleotide sequence shown in SEQ ID NO: 2; or

(2) a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence corresponding to a portion between nucleotide 77 and nucleotide 1270 of the nucleotide sequence shown in SEQ ID NO: 2, and encoding a protein having O-glycan α2,8-sialyltransferase activity.

[0010]

In another aspect of the present invention, the followings are provided: a recombinant vector (preferably, an expression vector) comprising the above-described O-glycan α2,8-sialyltransferase gene of the present invention; a transformant transformed with the above recombinant vector; and a method for producing the enzyme of the present invention wherein the above transformant is cultured and the enzyme of the present invention is collected from the culture.

[0011]

In another aspect of the present invention, a protein which comprises an active domain of O-glycan α2,8-sialyltransferase having any one of the following amino acid sequences is provided:

(1) an amino acid sequence corresponding to a portion between positions 26 and 398 of the amino acid sequence shown in SEQ ID NO: 1; or

(2) an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence corresponding to a portion between positions 26 and 398 of the amino acid sequence shown in SEQ ID NO: 1, and having O-glycan α2,8-sialyltransferase activity.

[0012]

In another aspect of the present invention, an extracellular secretory protein is provided, which comprises a polypeptide portion of the active domain and a signal peptide of the O-glycan α2,8-sialyltransferase of the present invention, and has O-glycan α2,8-sialyltransferase activity.

In another aspect of the present invention, a gene encoding the above-described extracellular secretory protein of the present invention is provided.

[0013]

In another aspect of the present invention, the followings are provided: a recombinant vector (preferably, an expression vector) comprising a gene encoding the above-described extracellular secretory protein of the present invention; a transformant

transformed with the above recombinant vector; and a method for producing the protein of the present invention wherein the above transformant is cultured and the enzyme of the present invention is collected from the culture.

[0014]

[Mode for Carrying out the Invention]

The embodiments of the present invention and the methods for carrying out the present invention will be described in detail below.

(1) Enzyme and protein of the present invention

The O-glycan α2,8-sialyltransferase of the present invention is characterized in that it has the following substrate specificity and substrate selectivity.

Substrate specificity: the substrates of the enzyme are glycoconjugates having a Siaα2,3(6)Gal structure (wherein Sia represents sialic acid and Gal represents galactose) at the terminus thereof.

Substrate selectivity: the enzyme incorporates sialic acids into O-glycan more preferentially than into glycolipids or N-glycans.

[0015]

The above-described substrate specificity and substrate selectivity are characteristics which have been demonstrated by mouse-derived O-glycan α2,8-sialyltransferases obtained in examples described in the present specification. The O-glycan α2,8-sialyltransferase of the present invention is not only derived from a mouse, and it is easily understandable for a person skilled in the art that the same type of O-glycan α2,8-sialyltransferase exists in the tissues of other mammals and that those O-glycan α2,8-sialyltransferases have a high homology to one another.

[0016]

Such O-glycan α2,8-sialyltransferases are characterized in that they have the above-described substrate specificity and substrate selectivity. These enzymes are also included in the scope of the present invention.

Examples of such an O-glycan α2,8-sialyltransferase may include natural enzymes derived from mammalian tissues and mutants thereof, and extracellular secretory proteins catalyzing the transfer of sialic acid to O-glycans through an α2,8-linkage, which are produced by genetic recombination, such as those produced in examples described later. These are also included in the scope of the present invention.

[0017]

O-glycan α2,8-sialyltransferase having either one of the following amino acid sequences may be one example of the O-glycan α2,8-sialyltransferase of the present invention:

(1) an amino acid sequence shown in SEQ ID NO: 1; or

(2) an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, and having O-glycan α2,8-sialyltransferase activity.

[0018]

In addition, it is to be understood that an active domain of the O-glycan α2,8-sialyltransferase of the present invention and proteins having O-glycan α2,8-sialyltransferase activity obtained by alteration or modification of a portion of the amino acid sequence thereof are all included in the scope of the present invention. Preferred examples of such an active domain may include an active domain of O-glycan α2,8-sialyltransferase corresponding to a portion between positions 26 and 398 of the amino acid sequence shown in SEQ ID NO: 1. A sequence portion between positions 26 and approximately 100 of the amino acid sequence shown in SEQ ID NO: 1 is a region called stem, and it is considered that this region is not necessarily required for the activity. Accordingly, a region corresponding to positions 101 to 398 of the amino acid sequence shown in SEQ ID NO: 1 may be used as an active domain of O-glycan α2,8-sialyltransferase.

[0019]

That is to say, the present invention provides a protein which comprises an active domain of O-glycan α2,8-sialyltransferase having any one of the following amino acid sequences:

(1) an amino acid sequence corresponding to a portion between positions 26 and 398 of the amino acid sequence shown in SEQ ID NO: 1; or

(2) an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence corresponding to a portion between positions 26 and 398 of the amino acid sequence shown in SEQ ID NO: 1, and having O-glycan α2,8-sialyltransferase activity.

[0020]

In the present specification, the range of "one or several" in the expression "an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids" is not particularly limited. For example, it means 1 to 20 amino acids, preferably 1 to 10 amino acids, more preferably 1 to 7 amino acids, further more preferably 1 to 5 amino acids, and particularly preferably 1 to 3 amino acids.

[0021]

A method for obtaining the enzyme or protein of the present invention is not particularly limited. The protein of the present invention may be a protein synthesized by chemical synthesis, or recombinant protein produced by genetic recombination.

When a recombinant protein is produced, first, DNA encoding the protein is required to be obtained. Suitable primers are designed based on the information regarding amino acid sequence shown in SEQ ID NO: 1 and nucleotide sequence shown in SEQ ID NO: 2 of the sequence listing in the present specification. Thereafter, using the obtained primers, PCR is carried out with a suitable cDNA library as a template, so as to obtain DNA encoding the enzyme of the present invention.

For example, a method for isolating cDNA encoding O-glycan α2,8-sialyltransferases having amino acid sequences shown in SEQ ID NO: 1 is

described in detail in examples described later. However, a method for isolating cDNA encoding the O-glycan α2,8-sialyltransferase of the present invention is not limited thereto. A person skilled in the art could easily isolate cDNA of interest by referring to the methods described in examples below and appropriately modifying or altering them.

[0022]

Moreover, when a partial fragment of DNA encoding the enzyme of the present invention is produced by the above-described PCR, the produced DNA fragments can be successively ligated to one another, so as to obtain DNA encoding a desired enzyme. The obtained DNA can be then introduced into a suitable expression system, so as to generate the enzyme of the present invention. Expression of the enzyme in such an expression system will be described later in the specification.

[0023]

An extracellular secretory protein, which comprises a polypeptide portion of the active domain of the O-glycan α2,8-sialyltransferase of the present invention and a signal peptide, and has O-glycan α2,8-sialyltransferase activity is also included in the present invention.

[0024]

In some cases, the O-glycan α2,8-sialyltransferase of the present invention may remain in cells after the expression and may not be secreted outside of the cells. In addition, there is a possibility that the production of the enzymes may be decreased when the intracellular concentration thereof exceeds a certain limit. In order to effectively use the activity of the above O-glycan α2,8-sialyltransferase to transfer sialic acid to O-glycans through an α2,8-linkage, a soluble form of proteins retaining the activities of the present enzymes and being secreted from cells during the expression may be produced. An example of such a protein may be an extracellular secretory protein, which comprises a signal peptide and a polypeptide portion of the active domain of O-glycan α2,8-sialyltransferase which is involved in the activity of the O-glycan

α2,8-sialyltransferase of the present invention, and catalyzes the transfer of sialic acid to O-glycans through an α2,8-linkage. For example, a fusion protein with protein A as desribed in the specification is preferred embodiments of the secretory protein of the present invention.

[0025]

Sialyltransferases that have been cloned so far have a domain structure similar to that of other glycosyltransferases. This is to say, the previously cloned sialyltransferases comprise an NH_2-terminal short cytoplasmic tail, a hydrophobic signal anchor domain, a stem region having proteolytic sensitivity, and a COOH-terminal large active domain (Paulson, J.C. and Colley, K.J., *J. Biol. Chem.*, 264, 17615-17618, 1989). In order to examine the position of a transmembrane domain of the O-glycan α2,8-sialyltransferase of the present invention, a hydropathy plot prepared according to the method of Kyte and Doolittle (Kyte, J. and Doolittle, R.F., *J. Mol. Biol.*, 157, 105-132, 1982) can be used. Moreover, in order to estimate an active domain portion, recombinant plasmids into which various types of fragments are introduced are produced and used. An example of such methods is described in detail, for example, in PCT/JP94/02182. However, a method for confirming the position of a transmembrane domain or estimating an active domain portion is not limited thereto.

[0026]

In order to produce an extracellular secretory protein which comprises a polypeptide portion of the active domain of O-glycan α2,8-sialyltransferase and a signal peptide, for example, a sequence corresponding to the active domain of O-glycan α2,8-sialyltransferase may be subjected to inframe fusion with an immunoglobulin signal peptide sequence as a signal peptide. As such a method, the method of Jobling (Jobling, S.A. and Gehrke, L., *Nature* (Lond.), 325, 622-625, 1987), for example, can be used. Further, as is described in detail in examples of the present specification, a fusion protein with protein A may also be produced. However, the type of a signal peptide, the

method of the fusion of a signal peptide with an active domain, and the method of solubilization are not limited to those described above. A person skilled in the art may appropriately select a polypeptide portion which is an active domain of O-glycan α2,8-sialyltransferase, and may fuse the selected polypeptide portion with any available signal peptide by a suitable method, so as to produce an extracellular secretory protein.

[0027]

(2) Gene of the present invention

The present invention provides a gene encoding the amino acid sequence of the O-glycan α2,8-sialyltransferase of the present invention.

Specific examples of a gene encoding the amino acid sequence of the O-glycan α2,8-sialyltransferase of the present invention may include genes having any one of the following nucleotide sequences:

(1) a nucleotide sequence corresponding to a portion between nucleotide 77 and nucleotide 1270 of a nucleotide sequence shown in SEQ ID NO: 2; or

(2) a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence corresponding to a portion between nucleotide 77 and nucleotide 1270 of the nucleotide sequence shown in SEQ ID NO: 2, and encoding a protein having O-glycan α2,8-sialyltransferase activity.

[0028]

The range of "one or several" in the expression "a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides" in the present specification is not particularly limited. For example, it means 1 to 60 nucleotides, preferably 1 to 30 nucleotides, more preferably 1 to 20 nucleotides, further more preferably 1 to 10 nucleotides, further more preferably 1 to 5 nucleotides, and particularly preferably 1 to 3 nucleotides.

[0029]

A gene encoding a protein comprising an active domain of the O-glycan α2,8-sialyltransferase of the present invention, and a gene encoding an extracellular secretory protein which comprises a polypeptide portion which is the above active domain and a signal peptide and has O-glycan α2,8-sialyltransferase activity, are also included in the scope of the present invention.

[0030]

The gene of the present invention can be obtained by the above-described method.

A method of introducing a desired mutation into a certain nucleic acid sequence is known to those skilled in the art. For example, known techniques such as site-directed mutagenesis, PCR using degenerated oligonucleotides, or exposure of cells containing nucleic acid to a mutagenic agent or radioactive ray are used as appropriate, whereby DNA comprising a mutation can be constructed. Such known techniques are described, for example, in Molecular Cloning: A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.,1989; and Current Protocols in Molecular Biology, Supplements 1 to 38, John Wiley & Sons (1987-1997).

[0031]

(3) Recombinant vector of the present invention

The gene of the present invention can be inserted into a suitable vector and used. The type of a vector used in the present invention is not particularly limited. For example, it may be autonomously replicating vector (e.g., a plasmid, etc.), or it may be a vector which is incorporated into the genome in host cells when it is introduced into the host cells, and replicates with an incorporated chromosome.

The vector used in the present invention is preferably an expression vector. In an expression vector, elements necessary for transcription (e.g., a promoter, etc.) are functionally ligated to the gene of the present invention. A promoter is a DNA sequence having transcription activity in host cells, and it can appropriately be selected depending on the type of host cells.

[0032]

Examples of a promoter capable of functioning in bacterial cells may include a *Bacillus stearothermophilus* maltogenic amylase gene promoter, a *Bacillus licheniformis* alpha-amylase gene promoter, a *Bacillus amyloliquefaciens* BAN amylase gene promoter, a *Bacillus subtilis* alkaline protease gene promoter, a *Bacillus pumilus* xylosidase gene promoter, a phage λ P_R or P_L promoter, and an *Escherichia coli* lac, trp, or lac promoter.

[0033]

Examples of a promoter capable of functioning in mammalian cells may include an SV40 promoter, an MT-1 (metallothionein gene) promoter, and an adenovirus 2 major late promoter. Examples of a promoter capable of functioning in insect cells may include a polyhedrin promoter, a P10 promoter, an *Autographa californica* polyhedrosis basic protein promoter, a baculovirus immediate early gene 1 promoter, and a baculovirus 39K delayed-early gene promoter. Examples of a promoter capable of functioning in yeast host cells may include a promoter derived from a yeast glycolytic system gene, an alcohol dehydrogenase gene promoter, a TPI1 promoter, and an ADH2-4c promoter.

Examples of a promoter capable of functioning in filamentous cells may include an ADH3 promoter and a tpiA promoter.

[0034]

The DNA of the present invention may be functionally ligated to a human growth hormone terminator, or in the case where a host is Mycomycete, the DNA may be functionally ligated to an appropriate terminator such as a TPI1 terminator or ADH3 terminator, as necessary. The recombinant vector of the present invention may also comprise elements such as a polyadenylation signal (e.g., those derived from SV40 or adenovirus 5E1b region), a transcription enhancer sequence (e.g., SV40 enhancer), and a translation enhancer sequence (e.g., those encoding adenovirus VA RNA).

The recombinant vector of the present invention may further comprise a DNA sequence enabling the vector to replicate in host cells. An example may include an SV40 replication origin (when the host cells are mammalian cells).

[0035]

The recombinant vector of the present invention may further comprise a selective marker. Examples of a selective marker may include genes whose complements are deficient in host cells, such as dihydrofolate reductase (DHFR) or a *Schizosaccharomyces pombe* TPI gene, and drug resistant genes that are resistant to ampicillin, kanamycin, tetracycline, chloramphenicol, neomycin, hygromycin, etc.

A method of ligating the DNA of the present invention, a promoter, and a terminator and/or a secretory signal sequence, as desired, to one another, and inserting them into a suitable vector has been well known to those skilled in the art.

[0036]

(4) Transformant of the present invention, and production of protein using the same

The DNA or recombinant vector of the present invention can be introduced into a suitable host, so as to prepare a transformant.

Any cells may be used as host cells into which the DNA or recombinant vector of the present invention is introduced, as long as they allow the DNA construct of the present invention to express therein. Examples of host cells may include bacteria, yeasts, Mycomycetes, and higher eukaryotes.

[0037]

Examples of bacterial cells may include Gram-positive bacteria such as Bacillus or Streptomyces, and Gram-negative bacteria such as *Escherichia coli*. Transformation of these bacteria may be carried out by the protoplast method or known methods, using competent cells.

Examples of mammalian cells may include HEK293 cells, HeLa cells, COS cells, BHK cells, CHL cells, and CHO cells. A method of transforming mammalian cells and

allowing a DNA sequence introduced into the cells to express therein has also been known. Examples of such a method may include the electroporation, the calcium phosphate method, and the lipofection method.

[0038]

Examples of yeast cells may include cells belonging to Saccharomyces or Schizosaccharomyces. Examples of such cells may include *Saccharomyces cerevisiae* and *Saccharomyces kluyveri*. Examples of a method of introducing a recombinant vector into a yeast host may include the electroporation, the spheroplast method, and the lithium acetate method.

[0039]

Examples of other fungal cells may include cells belonging to filamentous fungi such as Aspergillus, Neurospora, Fusarium, or Trichoderma. When filamentous fungi are used as host cells, transformation can be carried out by incorporating a DNA construct into a host chromosome to obtain recombinant host cells. Such a DNA construct can be incorporated into a host chromosome according to known methods such as homologous recombination or heterologous recombination.

[0040]

When insect cells are used as host cells, a recombinant gene-introduced vector and baculovirus are co-introduced into insect cells, and recombinant virus is obtained in the culture supernatant of the insect cells. Thereafter, insect cells are infected with the recombinant virus, so that a protein is expressed (which is described in e.g. Baculovirus Expression Vectors, A Laboratory Manual; and Current Protocols in Molecular Biology, Bio/Technology, 6, 47 (1998)).

[0041]

As an example of baculovirus, *Autographa californica* nuclear polyhedrosis virus infecting Mamestra insects can be used.

Examples of insect cells used herein may include *Spodoptera frugiperda* ovarian cells Sf 9 and Sf21 [Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman and Company, New York (1992)], and *Trichoplusia ni* ovarian cells HiFive (manufactured by Invitrogen).

Examples of a method of co-introducing a recombinant gene-introduced vector and the above baculovirus into insect cells to prepare recombinant virus may include the calcium phosphate method and the lipofection method.

[0042]

The above transformant is cultured in a nutrient medium under conditions enabling the expression of the introduced DNA construct. In order to isolate and purify the enzyme of the present invention from the culture of the transformant, common protein isolation and purification methods may be applied.

For example, where the enzyme of the present invention is expressed in a state where it is dissolved in cells, the cells are recovered by centrifugation after completion of the culture, and they are then suspended in a water-type buffer solution. Thereafter, the cells were disintegrated with an ultrasonic disintegrator or the like, so as to obtain a cell-free extract. A purified sample can be obtained from a supernatant obtained by centrifuging the above cell-free extract, using singly or in combination the following common protein isolation and purification methods: solvent extraction method, salting-out using ammonium sulfate or the like, desalting, precipitation method using organic solvents, anion exchange chromatography using resin such as diethylaminoethyl (DEAE) sepharose, cation exchange chromatography using resin such as S-Sepharose FF (manufactured by Pharmacia), hydrophobic chromatography using resin such as butyl sepharose or phenyl sepharose, gel filtration using a molecular sieve, affinity chromatography, chromatofocusing, electrophoresis such as isoelectric focusing, etc.

The present invention will be further specifically described in the following examples. However, these examples are not intended to limit the scope of the present invention.

[0043]

[Examples]

The following reagents and samples were used in specific examples of the present invention. Fetuin, asialofetuin, bovine submaxillary mucin (BSM), α1-acid glycoprotein, ovomucoid, lactosyl ceramide (LacCer), GM3, GM1a, GD1a, GD1b, GT1b, CMP-NeuAc, 6'-sialyllactose, 3'-sialyl-*N*-acetyllactosamine, and Triton CF-54 were purchased from Sigma. 3'-sialyllactose and 6'-sialyl-*N*-acetyllactosamine were purchased from Calbiochem. *N*-acetylneuraminic acid (NeuAc), GM4, Gal, and *N*-acetylgalactosamine (GalNAc) were purchased from Wako Pure Chemical Industries, Ltd. GD3 was purchased from Snow Brand Milk Products Co., Ltd. GQ1b was purchased from Alexis Biochemicals. CMP-[^{14}C]-NeuAc (12.0 GBq/mmol) was purchased from Amersham Pharmacia Biotech. Sialidases (NANase II, III) were purchased from Glyko Inc. *N*-glycanase (Glycopeptidase F) was purchased from Takara Shuzo Co., Ltd. [α-^{32}P]dCTP was purchased from NEN. GM1b and its positional analogs, GSC-68, 2,3-sialylparagloboside (2,3-SPG), and 2,6-sialylparagloboside (2,6-SPG) were contributed from Prof. Makoto Kiso (Faculty of Agriculture, Gifu University). NeuAcα2,3Gal and NeuAcα2,6Gal were contributed from Dr. Hideki Ishida (The Noguchi Institute). An anti-GD3 monoclonal antibody KM641 was contributed from Dr. Kenya Shitara and Dr. Nobuo Hanai of Kyowa Hakko Kogyo Co., Ltd. Peroxidase-conjugated AffiniPure goat anti-mouse IgG + IgM (H + L) was purchased from Jackson Immuno Research. Desialylated (asialo) glycoproteins obtained by removing sialic acids from BSM, α1-acid glycoprotein, and ovomucoid were prepared by treating them at 80°C for 1 hour in 0.02 N HCl.

[0044]

Using the amino acid sequence of mouse sialyltransferase ST8Sia V, a clone encoding a novel sialyltransferase showing a homology with the above enzyme has been searched against the database of expressed sequence tag (dbEST) of the National Center for Biotechnology Information. As a result, clones deposited under GenBank™ accession Nos. BE633149, BE686184, and BF730564 were obtained. Based on the information regarding the nucleotide sequences of these clones, two types of synthetic DNA fragments, 5'-CTTTTCTGGAGAACTAAAGG-3' (corresponding to nucleotides 1001-1020 in Figure 1) (SEQ ID NO: 3) and 5'-AATTGCAGTTTGAGGATTCC-3' (corresponding to a complementary strand of nucleotides 1232-1251 in Figure 1) (SEQ ID NO: 4) were prepared. Thereafter, in accordance with the method of Israel (Israel, D. I. (1993) *Nucleic Acids Res.* **21**, 2627-2631), the cDNA library of each of mouse brain and heart was screened by the polymerase chain reaction method (PCR). As a result, a clone encoding a portion of a novel sialyltransferase was obtained from each cDNA library. In order to obtain a full-length clone, two types of synthetic DNA fragments 5'-TGGCTCAGGATGAGATCGGG-3' (corresponding to nucleotides 68-87 in Figure 1) (SEQ ID NO: 5) and 5'-TACTAGCGCTCCCTGTGATTGG-3' (corresponding to a complementary strand of nucleotides 725-746 in Figure 1) (SEQ ID NO: 6) were further prepared. Thereafter, using mouse kidney-derived cDNA as a template, DNA located between both the synthetic DNA fragments was amplified by PCR. The amplified fragment was ligated to a clone obtained from the mouse brain cDNA library, so as to obtain a full-length clone. This cDNA had a single open reading frame encoding type II transmembrane protein of 398 amino acids with an estimated molecular weight of 45,399. In addition, sialyl motifs conserved in sialyltransferases were present in the amino acid sequence thereof. This protein showed 42.0% and 38.3% homology with ST8Sia I and V, respectively, at an amino acid sequence level among known mouse sialyltransferases (Figure 2). As described below, since this protein had the activity of

α2,8-sialyltransferase, it was named as the *O*-glycan α2,8-sialyltransferase of the present invention, ST8Sia VI.

[0045]

In order to examine enzymatic properties of the protein, a secretory protein was produced. First, with regard to mouse ST8Sia VI, using two types of synthetic DNA fragments each containing a *Xho*I site, 5'-TGCTCTCGAGCCCAGCCGACGCGCCTGCCC-3' (corresponding to nucleotides 141-170 in Figure 1) (SEQ ID NO: 7) and 5'-TATTCTCGAGCTAAGAAACGTTAAGCCGTT-3' (corresponding to a complementary strand of nucleotides 1263-1293 in Figure 1) (SEQ ID NO: 8), a DNA fragment encoding the active domain of mouse ST8Sia VI was amplified by PCR with cloned full-length cDNA as a template. The amplified product was cleaved with *Xho*I, and a cleaved portion was inserted into the *Xho*I site of a mammalian expression vector, pcDSA. The obtained expression vector was named as pcDSA-ST8Sia VI. This encodes a secretory fusion protein comprising a signal peptide of mouse immunoglobulin IgM, *Staphylococcus aureus* protein A, and the active domain of ST8Sia VI. Using pcDSA-ST8Sia VI and lipofectamine (Invitrogen), transient expression was carried out in COS-7 cells (Kojima, N. *et al.* (1995) *FEBS Lett.* **360**, 1-4). The protein of the present invention secreted from the cell into which pcDSA-ST8Sia VI had been introduced was named as PA-ST8Sia VI. PA-ST8Sia VI was adsorbed to IgG-Sepharose (Amersham Pharmacia Biotech), and were then recovered from medium.

[0046]

Sialyltransferase activity was measured as follows according to the method of Lee et al. (Lee, Y.-C. *et al.* (1999) *J. Biol. Chem.* **274**, 11958-11967). A reaction solution (10 μl) containing 50 mM MES buffer (pH 6.0), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% Triton CF-54, 100 μM CMP-[^{14}C]-NeuAc, a glycoconjugate (which was added at 0.5 mg/ml in the case of glycolipids, and at 1 mg/ml in the case of glycoproteins or oligosaccharides),

and a PA-ST8Sia VI suspension, was incubated at 37°C for 3 to 20 hours. Thereafter, in the case of glycolipids, the reaction product was purified with a C-18 column (Sep-Pak Vac 100 mg; Waters) and the purified product was used as a sample, and in the case of oligosaccharides or glycoproteins, the reaction product was directly used as a sample. Thus, the obtained samples were subjected to analysis. In the case of oligosaccharides or glycolipids, the sample was spotted on a silica gel 60 HPTLC plate (Merck), and was then developed with a developing solvent consisting of ethanol : pyridine : n-butanol : water : acetic acid = 100 : 10 : 10 : 30 : 3 (for oligosaccharides), or a developing solvent consisting of chloroform : methanol : 0.02% $CaCl_2$ = 55 : 45 : 10 (for glycolipids). In the case of glycoproteins, analysis was carried out by SDS-polyacrylamide gel electrophoresis. The obtained radioactivities were visualized with a BAS2000 radio image analyzer (Fuji Film) and then quantified.

Table 1 shows substrate specificity of PA-ST8Sia VI.

[0047]

[Table 1]

Table 1 Acceptor substrate specificity of ST8Sia VI

Using PA-ST8Sia VI, specificity against various acceptor substrates was examined. The concentration of the substrates is 0.5 mg/ml in the case of glycolipids, and 1 mg/ml in the case of glycoproteins, monosaccharides and oligosaccharides. The relative activity was calculated by taking incorporation obtained with Fetuin (2.06 pmol/h/(ml enzyme solution)) as 100. R represents the remainder of the N-linked sugar chain.

Acceptor substrate	Representative structure	Relative activity (%)
glycoproteins		
Fetuin	NeuAcα2,3Galβ1,3GalNAc-*O*-Ser/Thr NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAc-*O*-Ser/Thr NeuAcα2,6(3)Galβ1,4GlcNAc-R	100
Asialofetuin		0
α1-Acid glycoprotein	NeuAcα2,6(3)Galβ1,4GlcNAc-R	0
Asialo- α1-Acid glycoprotein		0
BSM	NeuAcα2,6GalNAc-*O*-Ser/Thr GlcNAcβ1,3(NeuAcα2,6)GalNAc-*O*-Ser/Thr	375
Asialo-BSM		0
Ovomucoid	NeuAcα2,3Galβ1,4GlcNAc-R	6.2
Asialoovomucoid		0
glycolipids		
Lactosylceramide	Galβ1,4Glcβ1-Cer	0
GM4	NeuAcα2,3Galβ1-Cer	1.0
GM3	NeuAcα2,3Galβ1,4Glcβ1-Cer	13.0
GM1a	Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1-Cer	0
GD1a	NeuAcα2,3Galβ1,3GalNAcβ1,4(NeuAca2,3)Galβ1,4Glcβ1-Cer	6.0
GD3	NeuAcα2,8NeuAcα2,3Galβ1,4Glcβ1-Cer	0
GD1b	Galβ1,3GalNAcβ1,4(NeuAcα2,8NeuAcα2,3)Galβ1,4Glcβ1-Cer	0
GT1b	NeuAcα2,8Galβ1,3GalNAcβ1,4(NeuAcα2,8NeuAcα2,3)Galβ1,4Glcβ1-Cer	1.1
GQ1b	NeuAcα2,8NeuAcα2,8Galβ1,3GalNAcβ1,4(NeuAcα2,8NeuAcα2,3)Galβ1,4Glcβ1-Cer	0
GM1b	NeuAcα2,3Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1-Cer	1.0
GSC-68	NeuAcα2,6Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1-Cer	2.6
2,3-SPG	NeuAcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1-Cer	3.5
2,6-SPG	NeuAcα2,6Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1-Cer	0.98
monosaccharides and oligosaccharides		
3'-Sialyllactose	NeuAcα2,3Galβ1,4Glc	629
6'-Sialyllactose	NeuAcα2,6Galβ1,4Glc	91.5
3'-Sialyl-*N*-acetyllactosamine	NeuAcα2,3Galβ1,4GlcNAc	411
6'- Sialyl-*N*-acetyllactosamine	NeuAcα2,6Galβ1,4GlcNAc	88.7
3'-Sialylgalactose	NeuAcα2,3Gal	13.9
6'-Sialylgalactose	NeuAcα2,6Gal	2.0
N-Acetylneuraminic acid	NeuAc	0
Galactose	Gal	0
N-Acetylgalactosamine	GalNAc	0

[0048]

PA-ST8Sia VI showed activity on glycolipids having a structure "NeuAcα2,3(6)Gal-" at the nonreducing end thereof, such as GM4, GM3, GD1a, GT1b, GM1b, GSC-68, 2,3-SPG, or 2,6-SPG. When GM3 was used as a substrate, the incorporated sialic acid of the reaction product was not cleaved with sialidase (NANase II), which specifically cleaves α2,3- and α2,6-linked sialic acid. However, the incorporated sialic acid was cleaved with sialidase (NANase III), which specifically cleaves α2,3-, α2,6-, α2,8- and α2,9-linked sialic acids (Figure 3A). In addition, it was confirmed by TLC immunostaining using an anti-GD3 monoclonal antibody KM641 (Saito, M. *et al.* (2000) *Biochim. Biophys. Acta* **1523**, 230-235) that this reaction product was GD3 into which sialic acid had been introduced through an α2,8-linkage (Figure 3B). Thus, it was clarified that PA-ST8Sia VI transfers sialic acid through an α2,8-linkage.

[0049]

On the other hand, where a glycoprotein was used as a substrate (Table 1), PA-ST8Sia showed the highest activity toward BSM, which contains only *O*-glycans as glycoconjugate. PA-ST8Sia also showed activity toward Fetuin, which contains both *O*-glycans and *N*-glycans and toward Ovomucoid, which contains only *N*-glycans. However, the activity toward Ovomucoid was lower than that toward a protein containing *O*-glycans. Moreover, PA-ST8Sia VI showed no activity on asialoglycoproteins. Furthermore, from an experiment wherein monosaccharide or oligosaccharide was used as a substrate (Table 1), it was found that the minimum sugar chain unit, which was recognized by PA-ST8Sia VI as a substrate, is NeuAcα2,3(6)Gal.

[0050]

It was found by an *N*-glycanase treatment that when Fetuin was used as a substrate, the majority of sialic acid, which was newly introduced by PA-ST8Sia VI, was incorporated into *O*-glycans (Figure 4). That is, when Fetuin was sialylated by PA-ST8Sia VI with [^{14}C]-NeuAc, and the sialylated product was then treated with

N-glycanase, which releases *N*-glycans from a peptide portion. The majority (82.7%) of radioactivity was still kept in the Fetuin after this treatment. This fact shows that the majority of sialic acid introduced by PA-ST8Sia VI was incorporated into *O*-glycans. On the other hand, the same experiment was carried out using ST8Sia III which used *N*-glycans as substrates. As a result, it was found that radioactivity completely disappeared.

[0051]

Moreover, in order to clarify the substrate specificity and substrate selectivity of PA-ST8Sia VI, the *Km* and *Vmax* values for BSM and GM3, respectively, were obtained. With regard to BSM, the *Km* value was 0.03 mM, the *Vmax* value was 23.8 pmol/h/ml enzyme solution, and the *Vmax/Km* value was 793. With regard to GM3, the *Km* value was 0.5 mM, the *Vmax* value was 0.67 pmol/h/ml enzyme solution, and the *Vmax/Km* value was 1.34. These results show that, for PA-ST8Sia VI, *O*-glycans are much more preferable substrates than glycolipids or *N*-glycans. Accordingly, it can be said that ST8Sia VI had substrate specificity different from that of the conventional α2,8-sialyltransferases.

[0052]

[Effect of the Invention]

The present invention provides a novel enzyme O-glycan α2,8-sialyltransferase, and a novel protein having an active portion of the enzyme and being extracellularly secreted. The enzyme and protein of the present invention have the activity of O-glycan α2,8-sialyltransferase. Accordingly, it is useful as a reagent for introducing a human-type sugar chain into a protein, for example. In addition, the O-glycan α2,8-sialyltransferase of the present invention is useful also as a medicament for treating hereditary diseases caused by deficiency of sugar chains specific for humans. Moreover, the O-glycan α2,8-sialyltransferase of the present invention can also be used as a medicament which acts for suppression of cancer metastasis, prevention of virus

infection, suppression of inflammatory response, or activation of neural cells. Furthermore, the O-glycan α2,8-sialyltransferase of the present invention is useful also as a reagent used in studies for increasing physiological action by adding sialic acid to drugs or the like.

[0053]

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[Brief Description of the Drawings]

[Fig.1]

Figure 1 shows the nucleotide sequences of ST8Sia VI cDNA, and the deduced amino acid sequences. A transmembrane domain is underlined, sialyl motif L is double-underlined, and sialyl motif S is dashed-underlined. Histidine and glutamic acid, which are conserved in sialyl motif VS, are boxed. Asparagine residues of the potential N-linked glycosylation sites are overlined.

[Fig.2]

Figure 2 shows a comparison made among the amino acid sequences of mouse sialyltransferases ST8Sia I, ST8Sia V, and ST8Sia VI. The conserved amino acid residues among these sialyltransferases are boxed. Sialyl motif L is duble-underlined, and sialyl motif S is dashed-underlined. The conserved histidine and glutamic acid residues in sialyl motif VS are marked with asterisks.

[Fig.3]

Figure 3 shows an analysis of linkage of sialic acid which was introduced into GM3 by PA-ST8Sia VI, and a TLC immunostaining of the reaction product.

A, GM3 was sialylated with [^{14}C]-NeuAc by PA-ST8Sia VI, and was treated with α2,3-, and α2,6-linkage specific sialidase (NANase II) or with α2,3-, α2,6-, α2,8-, and α2,9-linkage specific sialidase (NANase III), and then the reaction products were analyzed by HPTLC. The obtained results are shown.

B, GM3 was sialylated by PA-ST8Sia VI, and the reaction product was analyzed by TLC immunostaining. The obtained results are shown. Lane 1, GD3 (1 μg); lane 2, GM3 (1 μg); and lane 3, the reaction product. The reaction product was reacted with an

anti-GD3 monoclonal antibody KM641 and peroxidase-conjugated anti-mouse IgG + IgM (H+L) antibody, and then detected using an ECL system.

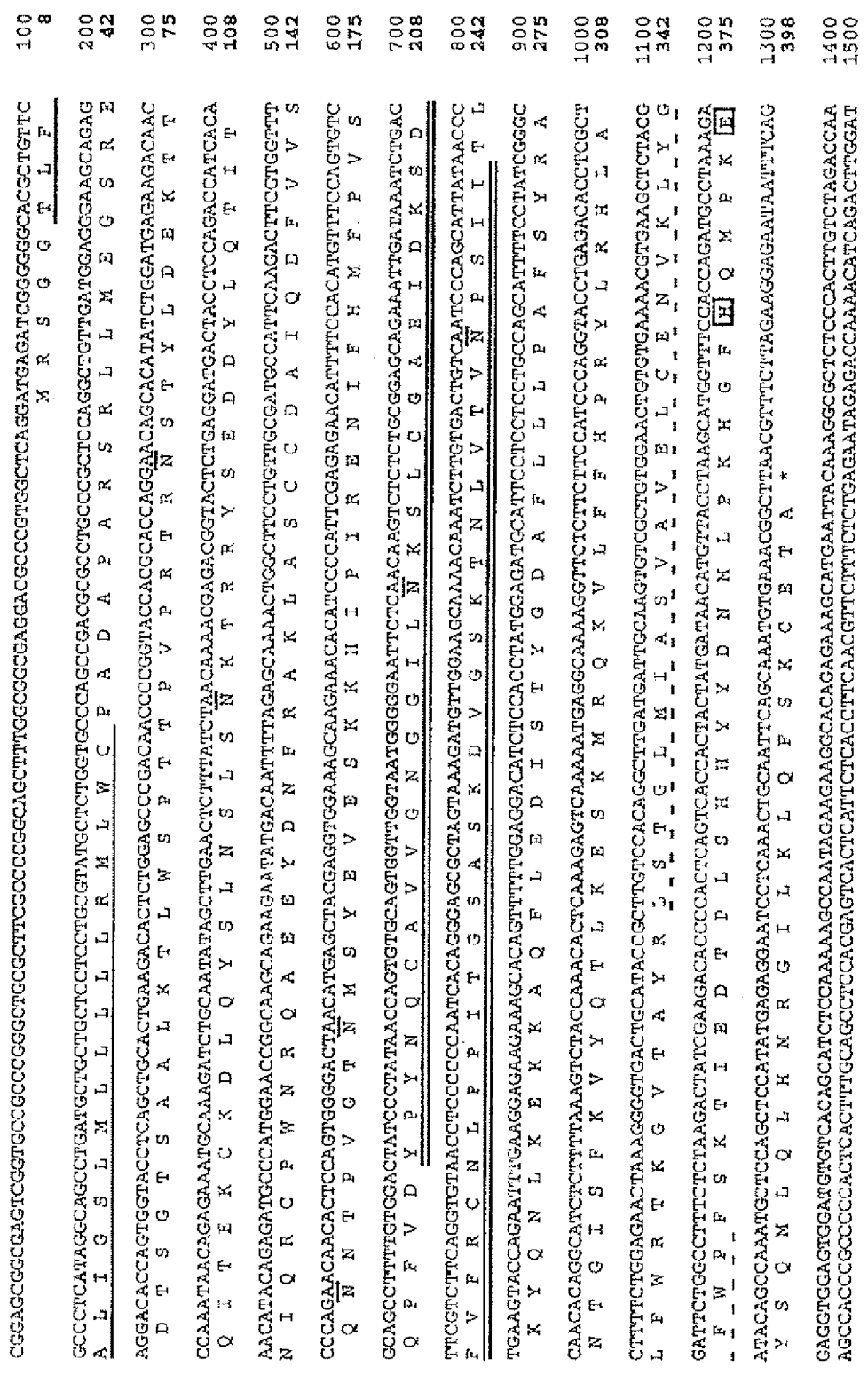
[Fig.4]

In Figure 4, Fetuin was [^{14}C]-NeuAc-incorporated by ST8Sia III or ST8Sia VI and then treated with *N*-glycanase.

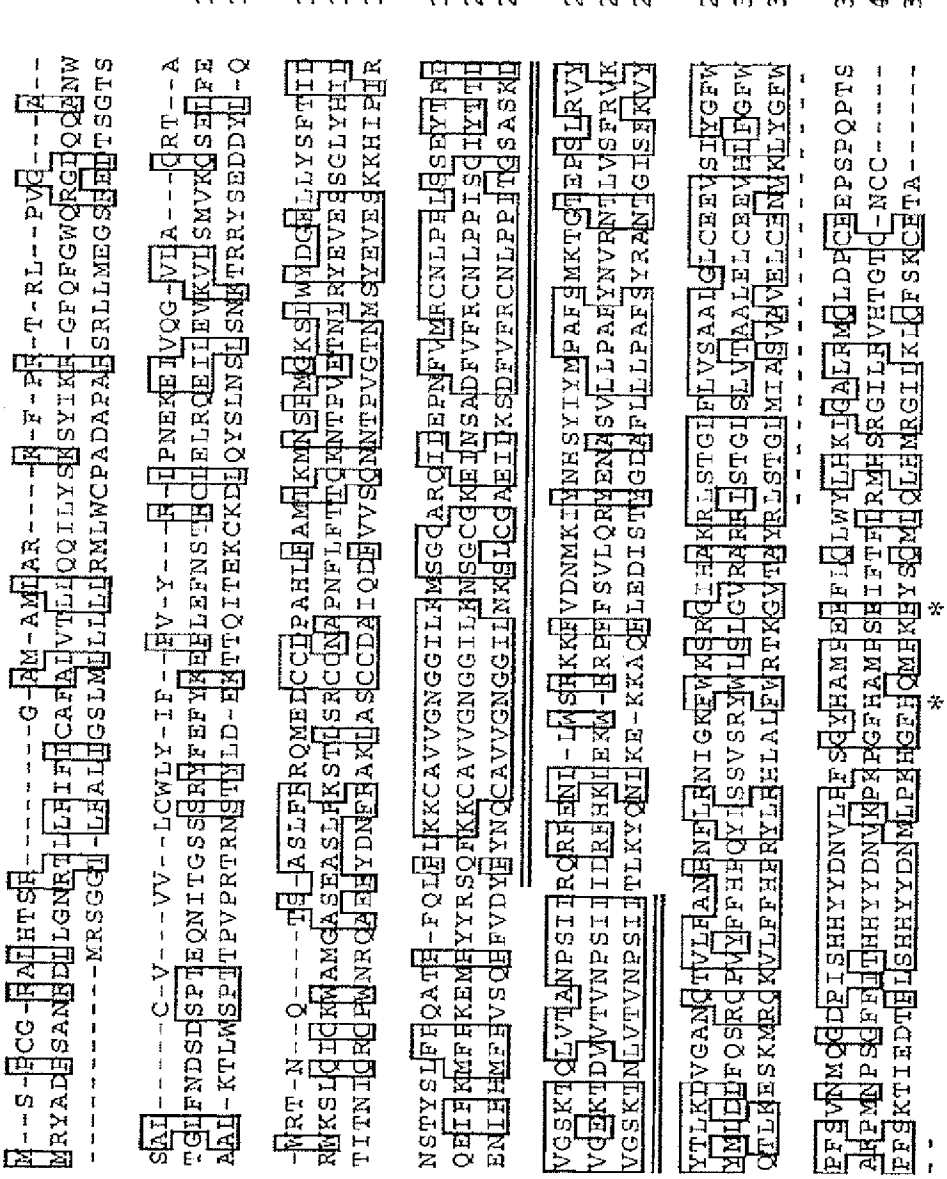
The [^{14}C]-NeuAc-incorporated Fetuin was treated with *N*-glycanase, and the treated product was analyzed by SDS-PAGE. Thereafter, it was visualized with a BAS2000 radio image analyzer.

[Name of Document] DRAWING

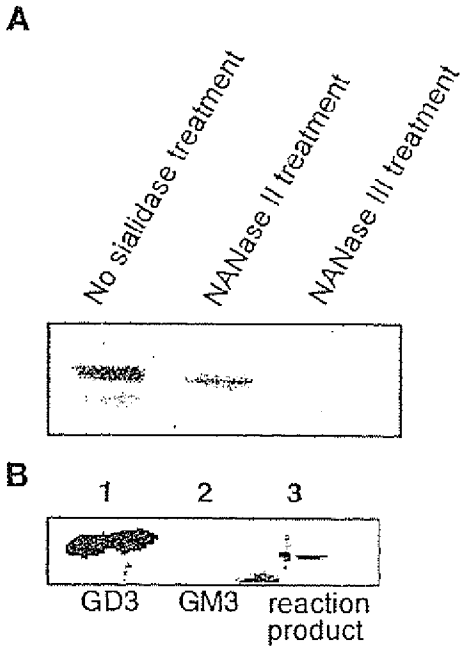
[Fig.1]



[Fig.2]



[Fig.3]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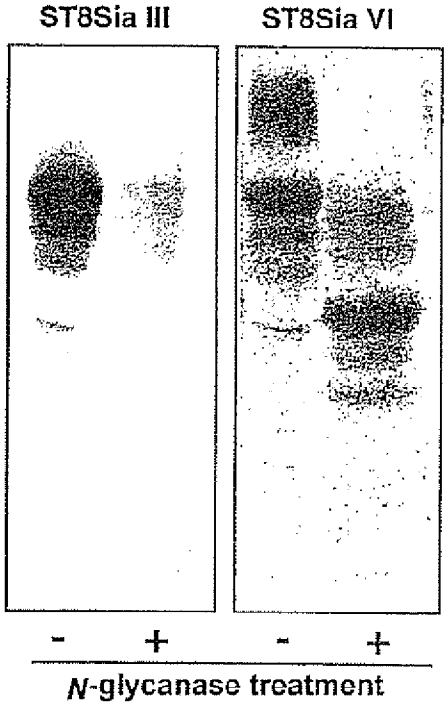
NANase II treatment
NANase III treatment
B
1
2
3
GD3
GM3
reaction product

[Fig.4]


ST8Sia III
ST8Sia VI
-
+
-
+
N-glycanase treatment

[Name of Document] ABSTRACT

[Abstract]

[Object] To provides a novel O-glycan α2,8-sialyltransferase showing high activity toward O-glycans.

[Means for Solution] O-glycan α2,8-sialyltransferase, which is characterized in that it has the following substrate specificity and substrate selectivity.

Substrate specificity: the substrates of the enzyme are glycoconjugates having a Siaα2,3(6)Gal structure (wherein Sia represents sialic acid and Gal represents galactose) at the terminus thereof.

Substrate selectivity: the enzyme incorporates sialic acids into O-glycans more preferentially than into glycolipids or N-glycans.

[Selected Drawing] None